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Molecular-Biochemical Identification and Antibiotic Sensitivity of Pathogenic Bacteria Isolated from Diseased Gangetic Mystus (*Mystus cavasius*, Hamilton 1822) in Biofloc Aquaculture

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A B S T R A C T

Biofloc technology has shown more production compared to the conventional fish culture but disease outbreaks have been seen to impede productivity. To identify pathogenic bacteria from diseased Gangetic Mystus (*Mystus cavasius*), using molecular, and biochemical techniques and to find out effective antibiotics. Bacterial samples from diseased fishes were cultured on nutrient agar and Rimler-Shotts (R-S) media. Various Biochemical tests like Gram's Staining, Triple Sugar Iron test, Catalase test, Motility test, Carbohydrate Fermentation test, Indole test, Methylred and Voges-Proskaur (MR-VP) test were performed. Sensitivity of isolated bacteria was tested against 15 commercially available antibiotics. Colony Characteristics from both nutrient agar and R-S media and the results of biochemical tests indicated that the suspected pathogens were *Aeromonas* spp. The obtained 16S rRNA gene sequence (1410 bp) from *M. cavasius* confirmed that the isolated bacteria was *Aeromonas veronii* which showed 99.93% similarity with the complete genome of *A. veronii* (GenBank Accession number CP034967.1). Distinct clinical sings in the gill, kidney, spleen, and skin of *M. cavasius* were reliable indicators of bacterial pathogenicity. Among tested 15 antibiotics Streptomycin, Ciprofloxacin and Gentamycin were found more effective against *A. veronii*. The findings of this research will be helpful to identify specific bacteria and to control bacterial diseases of *M. cavasius*.

Introduction

Fisheries play a crucial role in providing nutritious animal protein, generating employment, and boosting economic growth worldwide (Gillanders and Begg, 2023). Aquaculture stands out as one of agriculture's fastest-growing sectors globally. Aquaculture, known for its affordability, is a vital dietary component, especially in low-income nations like Bangladesh, offering a rich source of protein, vitamins, and minerals (Naylor *et al.*, 2023). Fisheries significantly contributes to meeting the basic food needs of many, supplying protein to approximately 1 billion people globally (Fry *et al.*, 2019).

Gangetic Mystus (*Mystus cavasius*), locally known as Tengra, is a species of catfish found in South Asia. It is native to the freshwater rivers and streams of the Indian subcontinent, including India, Bangladesh, Nepal, and Pakistan (Mondal *et al.,* 2022). This species is particularly abundant in the Ganges, Brahmaputra, and Indus River basins. They are well-adapted to a variety of aquatic environments and can thrive in both natural and artificial water bodies (Gaffar *et al.,* 2023). It is relatively small, usually growing to lengths of about 15-20 centimeters (6-8 inches), although some individuals can reach up to 30 centimeters (12 inches) (Mondal *et al.,* 2022). *M. cavasius* is very unique for its variable diet preferences and opportunistic feeding behavior. Their primary food sources include aquatic insects, crustaceans, small fish, and detritus. They are known to be bottom feeders, using their barbells to scavenge for food in the substrate (Hossain, 2014). The conservation status of *M. cavasius* is generally considered to be of "Least Concern" (Chaklader *et al.,* 2014).

Fish become susceptible to these diseases when they experience biological imbalances, inadequate medical care, or stressors such as unfavorable environmental conditions or overpopulation (Assefa and Abunna, 2018). Some of the commonly encountered bacterial pathogens in fish include *Edwardsiella ictaluri* (Machimbirike *et al.,* 2022), *Flavobacterium columnare* (LaFrentz *et al.,* 2022), *Mycobacterium* sp. (Hashish *et al.,* 2018), *Aeromonas* spp. (Hayatgheib *et al.,* 2020) and so on. The future success of aquaculture hinges on effective management of water quality and the mitigation of fish diseases, both of which are significantly influenced by microscopic biota and various environmental variables. A considerable number of microorganisms responsible for diseases in farmed aquatic animals are widely distributed in nature, thriving in environments where favorable conditions for their growth exists (Mishra *et al.,* 2017). Consequently, fish farmers are increasingly disheartened by the challenges posed by bacterial infections in aquaculture (Rahman *et al.,* 2016). Appropriate control measures after proper diagnosis of the diseases with causative agents, using biochemical and molecular techniques, can ensure sustainable aquaculture production.

At the core of molecular pathogen identification lies the Polymerase Chain Reaction (PCR). PCR is an invaluable tool for amplifying specific DNA sequences unique to identify the pathogens present in fish samples (Yogananth *et al.,* 2009; Rahman *et al.,* 2021). This amplification process facilitates the detection and identification of various pathogens, spanning viruses, bacteria, and parasites. Moreover, DNA sequencing techniques enable researchers to delve into the genetic makeup of these pathogens (Aboyadak *et al.,* 2015). This, in turn, aids in the precise identification and classification of different pathogen strains or species. For a more quantitative assessment of infections, Quantitative PCR (qPCR) comes into play, enabling the measurement of pathogen DNA levels (Yogananth *et al.,* 2009; Morshdy *et al.,* 2022). Additionally, Next-Generation Sequencing (NGS) technologies empower researchers to sequence entire pathogen genomes, providing comprehensive insights into virulence factors and genetic diversity (Oliveira *et al.,* 2012). Fish diseases are one of the main constraints for successful implementation of intensive and semi-intensive aquaculture technologies (Hossain, 1995). A range of diseases could be found in farmed aquatic animals in Bangladesh (BFRI, 1999; Faruk *et al.,* 2004). Infectious diseases are decreasing production in aquaculture as well as open water capture in Haor region. Farmers are losing capital and thus they are losing interest in fish farming (Rahman *et al.,* 2015; Rahman *et al.,* 2018). Different chemicals including antibiotics are commonly used in large quantity in aquaculture for treatment of infectious diseases by the farmers, but they have no adequate knowledge about harmful effects of antibiotics and appropriate dosage of antibiotics or other aqua-drugs. Farmers often use excess amount of aqua-drugs due to influence of drug traders. There are several important concerns with regard to the use of chemicals in aquaculture (Subasinghe *et al.,* 2000). Some of these chemicals, especially antibiotics, are often non-biodegradable and persist in fish muscle and in the aquatic environment as residues. Thus, the use of unapproved drugs or misuse of approved drugs in aquaculture fish possesses a potential human health hazard.

Unfortunately, in depth research to diagnose diseases in fishes, especially Tengra (*M. cavasius*) from Biofloc aquaculture system, using modern techniques (e.g., culture of bacteria, PCR and Sequencing) and to prevent and control fish diseases is very limited. Little attention has also been paid on the study of effectiveness of approved antibiotics used in aquaculture industry in the country even though antibiotic resistance has become a great concern for human health welfare. Therefore, the study was conducted to identify infectious diseases of Tengra (*M. cavasius*) with causative agents through

sequencing of 16S rRNA gene of isolated bacteria and biochemical tests, and to find out appropriate antibiotics through Antibiotic Sensitivity Test using commonly used antibiotics against the isolated bacteria.

Materials and Methods

Sample Collection

Diseased Tengra, *Mystus cavasius* (Figure 1) were collected from Biofloc Fish Farms located in Sharail, Brahmanbaria, Bangladesh (Figure 2). The fishes were transported in live condition using oxygen bag. The study was performed during the period January 2022 to July 2022.

Isolation of Bacteria

Bacteria were collected from various infected organs i.e., Skin, gills, kidney and body cavity of the diseased *M. cavasius* and inoculated in Nutrient agar and Nutrient broth media. Then the media were incubated for 24 hours at 37°C in an Incubator. For pure culture, 9 ml steriled is tilled water was mixed with1ml nutrient broth culture. The mixture was shaken for 30 s at about 150 rpm. A series of dilutions of the suspension was made for each sample by adding 1ml diluted sample into 9 ml sterile distilled water and 1 ml sample from each test tube, and was placed into nutrient agar, allowing 24 hours incubation at 37°. A single suspected colony appeared in nutrient agar media. Then the colony was inoculated in Rimler-Shotts (R-S) Medium Base medium to confirm either the isolated bacteria is *Aeromonas* spp. or not. The R-S medium was prepared by adding 1.83g R-S medium base in 40ml distilled water and then 62 µlnovobiocin was addedin40mlRSmedia. The pure colony of the isolated bacteria was then inoculated in R-S medium and incubated for 12 hours at 37°C. The yellowish colour colonies on R-S medium confirmed the isolated bacteria could be *Aermonas* spp. The isolated bacteria was used for further biochemical and molecular identification.

Biochemical Identification of the Isolated Bacteria

Gram Staining

A bacterial smear was prepared by adding 1 drop of distilled water into the pure colony of the isolated bacteria on a slide and gentle heat (3 times/Second in the flame of the spirit lamp) was provided to fix the sample. The sample was stained with a few drops of Crystal violet for 1 minute and washed into running water for 2 seconds. Then the sample was stained with Gram iodine for 1 minute, Gram decolorizer for 10 seconds, and Safranin for 45-60 seconds respectively including rinsing in running water following each step. The slide was allowed to dry completely and observed the bacteria in100X using emersion oil. The purple color indicated that the isolated bacteria was Gram-positive or the Pink/red color indicated that the bacteria was Gram-negative bacteria.

Triple Sugar Iron Test

A media was prepared by adding 6.452g triple sugar iron (TSI) agar powder in 100 ml distilled water and heat to melt agar in water. Five test tubes were filled with 20 ml TSI agar media each and then the test tubes containing media were sterilized at 121°C with 15 pound pressure for 15 minutes in an autoclave and kept in a slant position to solidify. The isolated bacteria was stabbed in the butt and streaked on the slant and incubated for 24 hours at 37°C. The changes was observed in butt and slant: i) Slant red, Butt yellow, Black stab indicated Glucose+VE and H2S+VE; ii) Slant red, Butt yellow, no black stab indicated Glucose+VE and H2S–VE; iii) Slant and Butt yellow, Black Stab indicated Sucrose/Lactose+VE, H2S +VE; iv) Slant and Butt yellow, No Black stab indicated Sucrose/Lactose+VE and H2S–VE; and v) finally, Red Slant and Butt, no black Stab notified that the bacteria was Sugar–VE and H2S–VE.

Indole Test

The isolated bacteria was inoculated in 5 ml nutrient broth by sterile loop and incubated at 37°C for 48-72 hours. Xylene solution (1 ml) was added in the nutrient broth and finally, 0.5 ml Kovacs indole reagent was added as an indicator. The color changes were observed after 10-15 minutes: i) The pink color ring indicated indole positive and ii) No color changes indicated indole negative.

Catalase Test

The bacterial colony was placed on a slide by inoculating loop and then 1 drop of 30% hydrogen peroxide was placed onto the bacterial colony using a dropper. The

bubble formation was observed under the microscope. Bubble formation indicated catalase-positive and the absence of bubble indicated catalase-negative.

Methylred and Voges-Proskaur (MR-VP) test

MR-VP broth was prepared and distributed 5 ml into 4 test tubes each. The test tubes were autoclaved at 121°C and 15 pounds pressure for 15 minutes. The broth culture (5-6 drops) was added in each test tubes and incubated at 37°C for 48-72 hours. Then 3-5 drops of methylred indicator for MR test and 5 ml of 10% KOH for VP test were added and left open after shaking. The pink color within 15 minutes indicated MR positive and the pink color within 2, 12 and 24 hours indicated VP positive.

Carbohydrates fermentation test

The broth was prepared with 4 different CHO (sucrose, maltose, lactose, mannitol). The bacteria broth culture (5-6 drops) were inoculated into each of the 12 test tubes containing 3.5 ml of 1% of peptone water+1.5% of the sugar solution (sucrose, maltose, lactose, mannitol), and 2-3 drops of 1% bromocresol purple was added as an indicator and incubated at 37°C for 2-3 days. The Durham tube's trapped bubbles and the color changed to yellow indicating gas and acid production, respectively. A delayed fermentation resulted in orange color and no bubble in Durham tubes indicates an aerobic organisms.

Motility Test

A cavity slide was taken and placed on the table. A coverslip was prepared by petroleum jelly on the 4 corners. A small drop of bacterial suspension was placed into the center of the coverslip. The glass slide was put over the coverslip and turned it to make a hanging position. The hanging drop was observed in (100X) magnification using immersion oil to identify non-motile and motile bacteria.

Molecular Identification of the Isolated Bacteria

The DNA of *Aeromonas* sp. from pure culture of the isolated bacteria was extracted using Maxwell Blood DNA Kit (Model: AS1010, Origin: Promega, USA). PCR amplification of 16S rRNA gene was done using Forward primer: 27F (5´- AGA GTT TGA TCM TGG CTC AG-3´) and Reverse primer: 1492R (5´- CGG TTA CCT TGT TAC GAC TT-3´) in a final volume of 20 µL, utilizing Hot Start Green Master Mix composed of dNTPs, Buffer, MgCl2, and Taq Polymerase. The amplification protocol for 16S rRNA gene included 3 min denaturation at 95°C, then 30 s denaturation at 95°C (35 cycles), 30 s annealing at 48°C (35 cycles) and 90 s extension at 72°C (35 cycles), and finally 5 min extension at 72°C. The amplified PCR product was tested on agar gel using Gel electrophoresis, 100 bp DNA Ladder, 1kb DNA Ladder, Diamond™ Nucleic Acid Dye and TAE Buffer. Excess nucleotides and primers from PCR products were removed through purification process. After purification of the PCR product, the sequencing PCR was performed, using Big Dye Terminator kit and both the forward and the reverse primers. Then the DNA template was precipitated using ethanol. Finally sequencing was performed in a 3500xL Genetic Analyzer (AB).

Antibiotic Sensitivity Test

Broth culture of the isolated bacteria was spread simultaneously in petri-dishes containing Hinton-Mueller Agar (MHA) and incubated at 37°C for 24 hours. Then 15 commercially important antibiotics (Oxytetracycline - 30µg, Tetracycline - 30 µg, Ciprofloxacin-5 µg, Oxacillin- 1 µg, Ceftriaxone - 30 µg, Co-trimoxazole-25 µg, Doxycycline - 30µg, Levofloxacin-5 µg, Novobiocin- 30 µg, Erythromycin-15 µg, Amoxycillin- 30 µg, Ampicillin 20 µg, Azithromycin 15 µg, Streptomycin- 10 µg, and Gentamycin- 10 µg) were placed according to the disc diffusion methods and incubated 24 hours at 37°C. After the incubation period, the Zone of inhibition was measured to determine the Sensitive (≥18 mm), Intermediate (13–17 mm), and Resistant (≤18 mm) antibiotics.

Data and Sequence analysis

The obtained sequence of the isolated bacteria (*Aeromonas* spp.) from *M. cavasius* was edited and aligned with ClustalW Multiple Alignment using Bioedit (Hall, 1999), producing a 1410 bp long sequence. Basic Local Alignment Search Tool (BLAST) of NCBI (The National Center for Biotechnology Information) was used to analyze the sequence data. Thirty four 16S rRNA gene sequences of available *Aeromonas* spp. (*A. veronii* – 8, *A. caviae* – 8, *A. hydrophila* – 5, *A. enteropelogenes* – 2, *A. punctata* – 7, *A. taiwanensis* – 1, *A. dhakensis* - 3) were retrieved from GenBank and aligned with the sequence of this study, which produced a 1409 bp fragment. A maximum likelihood tree (Cladogram) was constructed using SeaView version 4.4.1 (Gouy *et al.*,

2010) based on Hasegawa-Kishino-Yano 85 (HKY85) model, derived from a PhyML Test (Guindon *et al.,* 2010) using the APE package (Paradis, 2006) in R (R Core Team, 2014) based on the lowest Akaike information criterion. MS Excel was used to analyze and show the antibiotic sensitivity data in bar diagram format.

Results and Discussion

Isolation and Identification

A single colony of the isolated bacteria grown on nutrient agar and R-S media in case of different diseased fishes was characterized as yellow in colour and round in shape, and thus the bacteria were suspected as *Aeromonas* sp. Based on the culture on nutrient agar and nutrient broth media, no other bacterial colonies were observed. A suspected pathogenic bacteria was isolated which was responsible for the infection in *M. cavasius*,

Morphological Characteristics of *Aeromonas* spp.

The bacteria isolated from the diseased fishes had shiny, spherical, convex, and creamy appearance (Figure 3; Table 1). Microscopically the isolated bacteria were Gram-negative single, paired, or in short-chain rod (Figure 3; Table 1).

Biochemical Identification

Gram Staining of *Aeromonas* spp.

Aeromonas spp. isolated from Tengra (*M. cavasius*) were found Gram-negative in Gram-Staining Test (Figure 3).

Triple Sugar Iron Test Result

Triple sugar iron test result of *Aeromonas* spp. showed the Slant changes to yellow and butt changes to yellow & blackened stab line. Triple sugar iron test result proved that the bacteria is sucrose/lactose positive and hydrogen sulfide positive.

Indole Test result

The pink color ring on the surface indicated that the bacteria isolated from Tengra (*M. cavasius*) were Indole positive (Figure 4).

Catalase Test Result

Formation of bubble during Catalase Test indicated the isolated bacteria *were* catalase-positive (Figure 5).

MR-VP Test Result

Methyl Red and Voges-Proskaure (MR-VP) test showed that the bacteria were MR negative VP negative for isolation from Tengra, *M. cavasius* (Figure 6).

Carbohydrates Fermentation Test Result

Color changes from red/purple to yellow indicates acid production and gas bubbles in Durham's tubes indicate gas production (Figure 7). The CHO fermentation result indicated that the isolated bacteria were sucrose, maltose, lactose, mannitol positive and produced acid and gas after the 72 hours incubation period (Figure 7).

Motility test result

In the hanging drop slide method it was found that the bacteria from Tengra (*M. cavasius*) were motile (Figure 8).

Colony Characteristics from both nutrient agar and R-S media and the results of biochemical tests (e.g., Gram-Staining test, Triple Sugar Iron Test, Indole Test, MR-VP Test, Carbohydrates Fermentation Test and Motility test) indicated that the suspected bacteria isolated from diseased Tengra (*M. cavasius*) was *Aeromonas* sp. Morphological and biochemical properties of the isolated *Aeromonas* sp. was very similar to those described by other scientists (Abbott *et al.,* 2003; Sabur, 2006; Mostafa *et al.,* 2008; Al-Fatlawy and Al-Hadrawy, 2014; Goni *et al.,* 2020; Parven *et al.,* 2020). Gram-negative bacteria are the most common cause of skin infections in freshwater fish (Yanong, 2011). Mass mortality in tilapia and all 20 bacterial isolates were Gram-negative and oxidation-positive (Abadi *et al.,* 2020). Catalase-positive was found in 11 of them, whereas catalase-negative was found in 9.

All of the Indole test reactions and bacterial carbohydrate fermentation were positive. Lactose positive is found in 12 isolates, whereas lactose negative is found in 8 isolates. *Aeromonas hydrophila* infection was blamed for the fish mortality in Bangladesh (Abadi *et al.,* 2020). *Aeromonas hydrophila* was reported as a Gram-negative

short plump rod which was motile and positive for oxidase and catalase test and produced acid and gas from a variety of sugar media, including lactose, glucose, sucrose, dextrose, maltose, and mannitol, but not inositol, sorbitol, or rhamnose (Monir *et al.,* 2017; Samal *et al.,* 2014; Noga, 2010).

Furthermore, *Aeromonas hydrophila* grew on citrate and produced acetone, indole, and reduce nitrate but showed a positive reaction to the Voges Proskauer (VP) test and a negative reaction to the methyl red (MR) test (Monir *et al.,* 2017; Samal *et al.,* 2014; Jayavignesh *et al.,* 2011; Mostafa and Ahamed, 2008).

Many researchers identified Motile aeromonads as potential threats to freshwater-farmed fish. Four bacteria (*Pseudomonas aeruginosa, Aeromonas veronii, Acinetobacter juvenii,* and *Citrobacter freundii*) were identified from infected Nile tilapia by several researchers (Dong *et al.,* 2015; Eissa *et al.,* 2015; Peepim *et al.,* 2016; Zhu *et al.,* 2016; Raj *et al.,* 2019). *Aeromonas* sp*, Escherichia* sp*., Vibrio* sp*., Pseudomonas* sp*., Salmonella* sp., and *Streptococcus* sp. were found in Nile tilapia farmed in Ethiopia (Bekele *et al.,* 2019).

Aeromonas sp. and *Pseudomonas* sp. were the most common bacterial pathogens worldwide, including Bangladesh and the present findings also suggest that *Aeromonas* is a potential threat to Nile tilapia cultured, especially in biofloc system (Hossian *et al.,* 2009). Summer mortalities in Egyptian farmed Nile tilapia (*O. niloticus*) were investigated and reported the fish having exophthalmia, external hemorrhagic patches, stomach distension, and skin discoloration (El Asely *et al.,* 2020). *Aeromonas* sp. has been associated with anorexia, gill and skin hemorrhages, weakness, and blindness (Yardimci and Aydin, 2011).

Morphological and biochemical properties of the isolated *Aeromonas* sp. were very similar to those described by other scientists (Abbott *et al.,* 2003; Al-Fatlawy and Al-Hadrawy, 2014; Mostafa *et al.,* 2008; Sabur, 2006). Gram-negative bacteria are the most common cause of skin infections in freshwater fish (Yanong, 2011). Mass mortality in tilapia and all 20 bacterial isolates were Gram-negative and oxidation-positive (Abadi *et al.,* 2020). Catalase-positive was found in 11 of them, whereas catalase-negative was found in 9. All of the Indole test reactions and bacterial carbohydrate fermentation were positive. Lactose positive is found in 12 isolates, whereas lactose negative is found in 8 isolates. *Aeromonas hydrophila* infection was blamed for the fish mortality in Bangladesh (Abadi *et al.,* 2020).

Aeromonas hydrophila was reported as a Gram-negative short plump rod which was motile and positive for oxidase and catalase test and produced acid and gas from a variety of sugar media, including lactose, glucose, sucrose, dextrose, maltose, and mannitol, but not inositol, sorbitol, or rhamnose (Monir *et al.,* 2017; Samal *et al.,* 2014; Noga, 2010). Furthermore, *Aeromonas hydrophila* grew on citrate and produced acetone, indole, and reduce nitrate but showed a positive reaction to the Voges Proskauer (VP) test and a negative reaction to the methyl red (MR) test (Monir *et al.,* 2017; Samal *et al.,* 2014; Jayavignesh *et al.,* 2011; Mostafa and Ahamed, 2008).

Molecular identification of the isolated bacteria using 16S rRNA sequence

The obtained 16S rRNA sequence (1410bps) of the bacteria isolated from diseased Tengra (*M. cavasius*) matched with 4656805 – 4658214 bps (Query cover-100% and Identity- 99.93%) of the 16S rRNA gene of *A. veronii* complete genome (GenBank Accession number: CP034967.1). The sequence also matched with *A. veronii* sequences, having GenBank accession numbers: MT345040.1 (Query cover 100%, Identity- 99.93%), MT026965.1 (Query cover- 100%, Identity- 99.93%), CP047155.1 (Query cover- 100%, Identity- 99.93%), CP046407.1 (Query cover- 100%, Identity- 99.93%), MN603643.1 (Query cover- 100%, Identity- 99.93%), MN519574.1 (Query cover- 100%, Identity- 99.93%), CPo44060.1 (Query cover- 100%, Identity- 99.93%), MK165135.1 (Query cover- 100%, Identity- 99.93%), and MN220509.1 (Query cover- 100%, Identity- 99.93%). Thus, the identity (Maximum 99.93%) of the sequence with the *A. veronii* sequences in NCBI confirmed that the bacteria isolated from *M. cavasius* was *A. veronii.* Thus, the current study first described the presence of *Aeromonas veronii* (strain BDMC_01) (GenBank Accession number: PP712114) in infected Tengra (*M. cavasius*) based on 16S rRNA sequence in Bangladesh. The molecular weight of the obtained sequence was 427439.00 daltons for single strand and nucleotide composition contains 55.60% G+C and 44.40% A+T (Figure 10). *Aeromonas veronii* was found responsible for mass mortality in biofloc system culturing Tengra (*M. cavasius*). The phylogenetic analysis also showed that the isolated bacteria of this study clustered together with other *A. veronii* sequences retrieved from GenBank and the cluster of the *A. veronii* is highly diverged from other *Aeromonas* spp. (Figure 9).

Table.1 Morphology and Gram staining result

Sample	Colony Characteristics	Shape	provisions	Gram staining reaction	Suspected bacterial group
Diseased Fish	Yellow-colored, round, and dense colony	Straight rods	Single paired, or in short chain	Gram-Negative	*Aeromonas* spp.

Table.2 Antibiogram of *Aeromonas veronii* isolated from diseased *M. cavasius*

SL No	Antibiotics	Abbreviation	Concentration	Diameter of Inhibition Zone (mm)	Susceptibility	Comments
1	Oxytetracycline	O	30µg/ml	70	+++	**Sensitive**
2	Tetracycline	TE	30µg/ml	50	+++	**Sensitive**
3	Ciprofloxacin	CIP	5µg/ml	100	+++	**Sensitive**
4	Oxacillin	OX	1µg/ml	0	-	**No effects**
5	Ceftriaxone	CTR	30µg/ml	10	+	**Resistant**
6	Co-trimoxazole	COT	25µg/ml	0	-	**No effects**
7	Doxycycline	DO	30µg/ml	70	+++	**Sensitive**
8	Levofloxacin	LE	5µg/ml	90	+++	**Sensitive**
9	Novobiocin	NV	30µg/ml	80	+++	**Sensitive**
10	Erythromycin	E	15µg/ml	80	+++	**Sensitive**
11	Amoxycillin	AMX	30µg/ml	40	+++	**Sensitive**
12	Ampicillin	A/S	20µg/ml	0	-	**No effects**
13	Azithromycin	AT	15µg/ml	60	+++	**Sensitive**
14	Streptomycin	S	10µg/ml	130	+++	**Sensitive**
15	Gentamicin	GEN	10µg/ml	100	+++	**Sensitive**

Note: (-) indicates: no inhibition and no effects against a bacterium; (+) indicates: inhibitory zone less than 13mm and antibiogram resistant; (++) indicates: the zone between 13- 17mm & intermediate effects on drugs; (+++) indicates: inhibitory zone ≥18mm and sensitivity to antibiogram

Figure.1 Diseased Tengra, *Mystus cavasius*: A. Distended abdomen (yellow arrow) of *M. cavasius*. B. Accumulation of abdominal fluid (white arrow), fin loss (red arrow). Exophthalmia (Pop eye) is visible in both fishes (small black arrow).

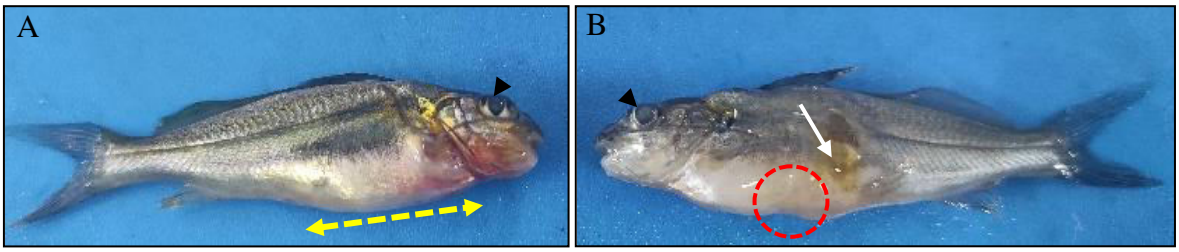


Figure.2 Sample collection area Sarail upazila, Brahmanbaria (circle).

Figure.3 Gram staining of the bacteria isolated from Tengra, *M. cavasius*

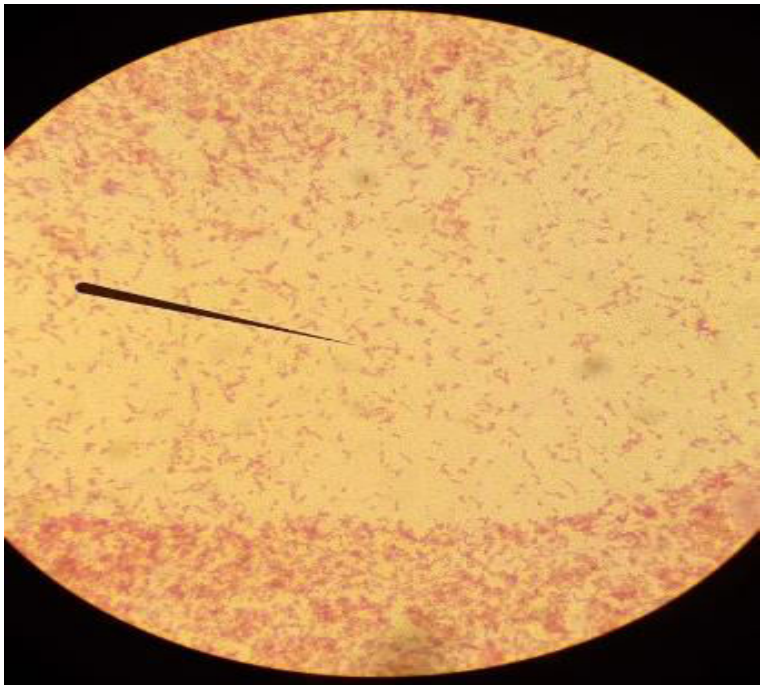


Figure.4 Indole Test of the bacteria isolated from Tengra, *M. cavasius*

Figure.5 Bubble formation during Catalase Test under the microscope for the isolated bacteria from Tengra, *M. cavasius*

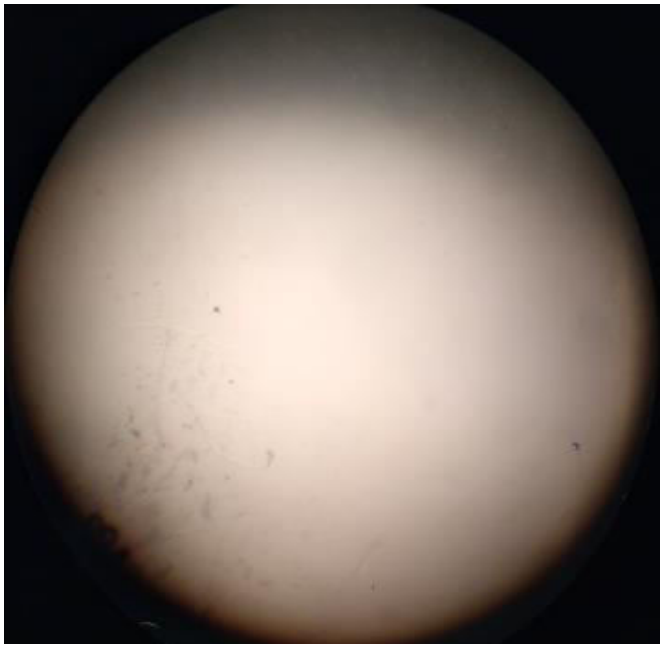


Figure.6 No color changes were observed for bacteria isolated from Tengra (*M. cavasius*) in the MR-VP Test

Figure.7 Observe the color changes and gas bubble production for the bacteria isolated from Tengra, *M. cavasius*

Figure.8 Microscopic observation of motility of the bacteria isolated from *M. cavasius*

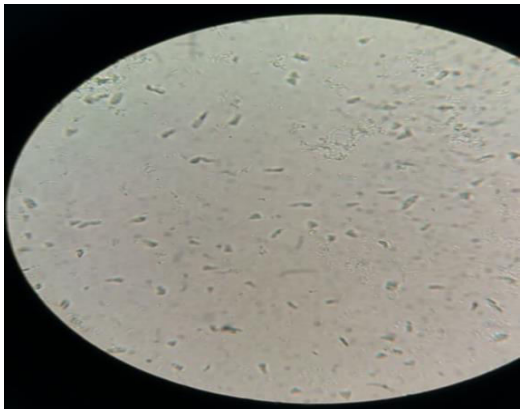
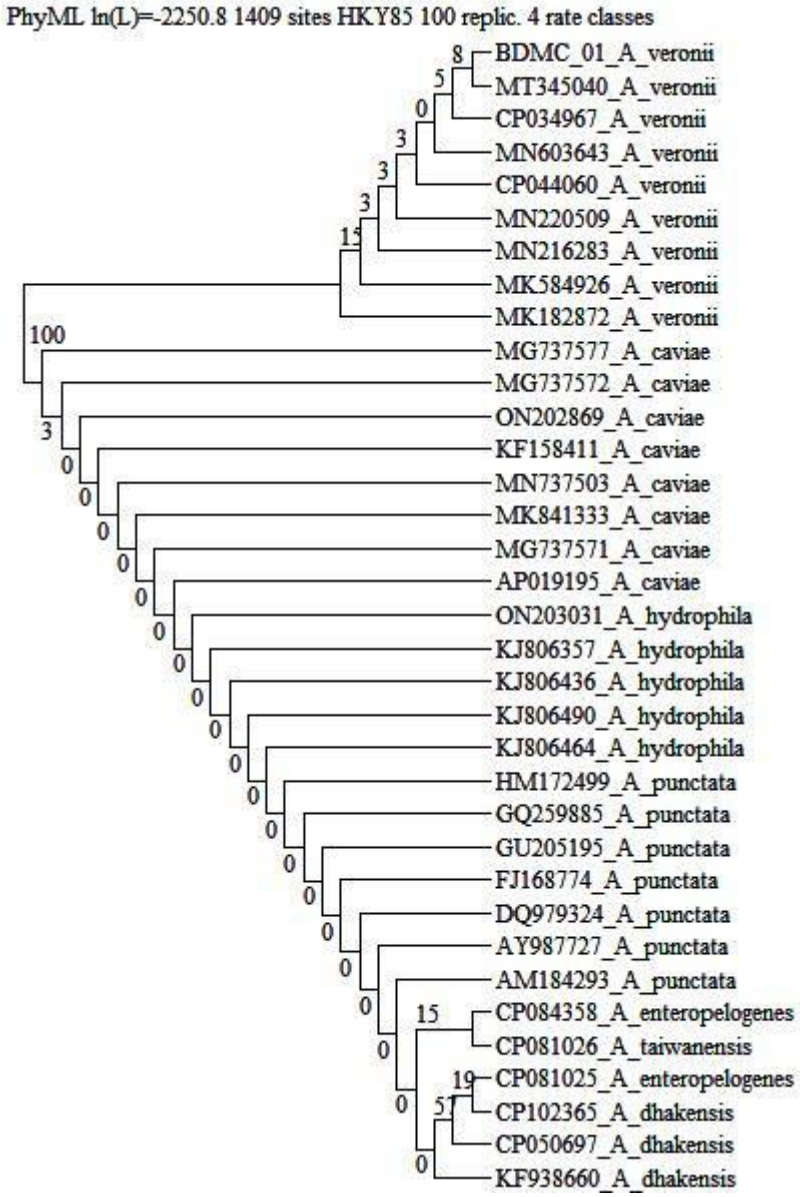


Figure.9 Maximum Likelihood Tree for *Aeromonas* spp. based on 16S rRNA. A 1409 bp fragment of 16S rRNA for 35 sequences were concatenated and the phylogeny was constructed under 'HKY85' model.



Abbreviations: BDMC_01_A_veronii isolated from Bangladesh *Mystus cavasius*. The numbers accompanied the *Aeromonas* spp. represents the respective GenBank accession numbers.

Figure.10 Nucleotide compositions of *Aeromonas veronii* isolated from diseased *M. cavasius*

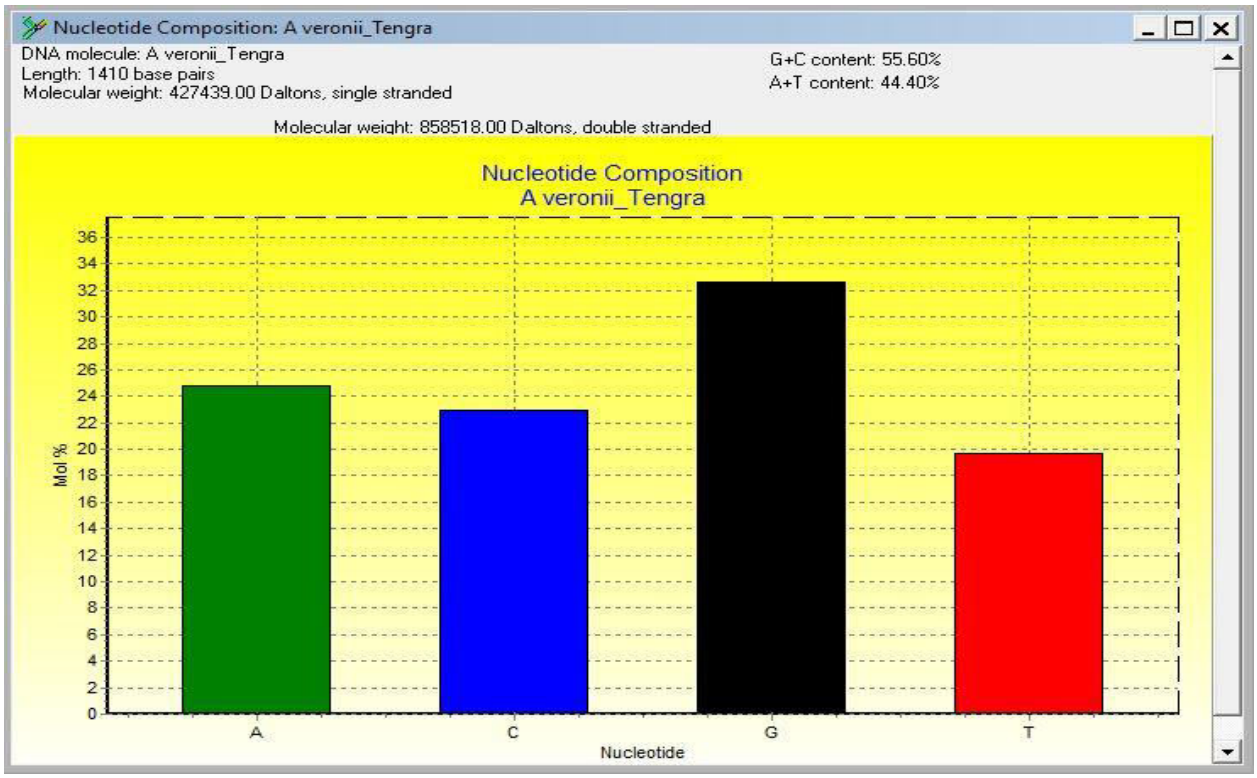
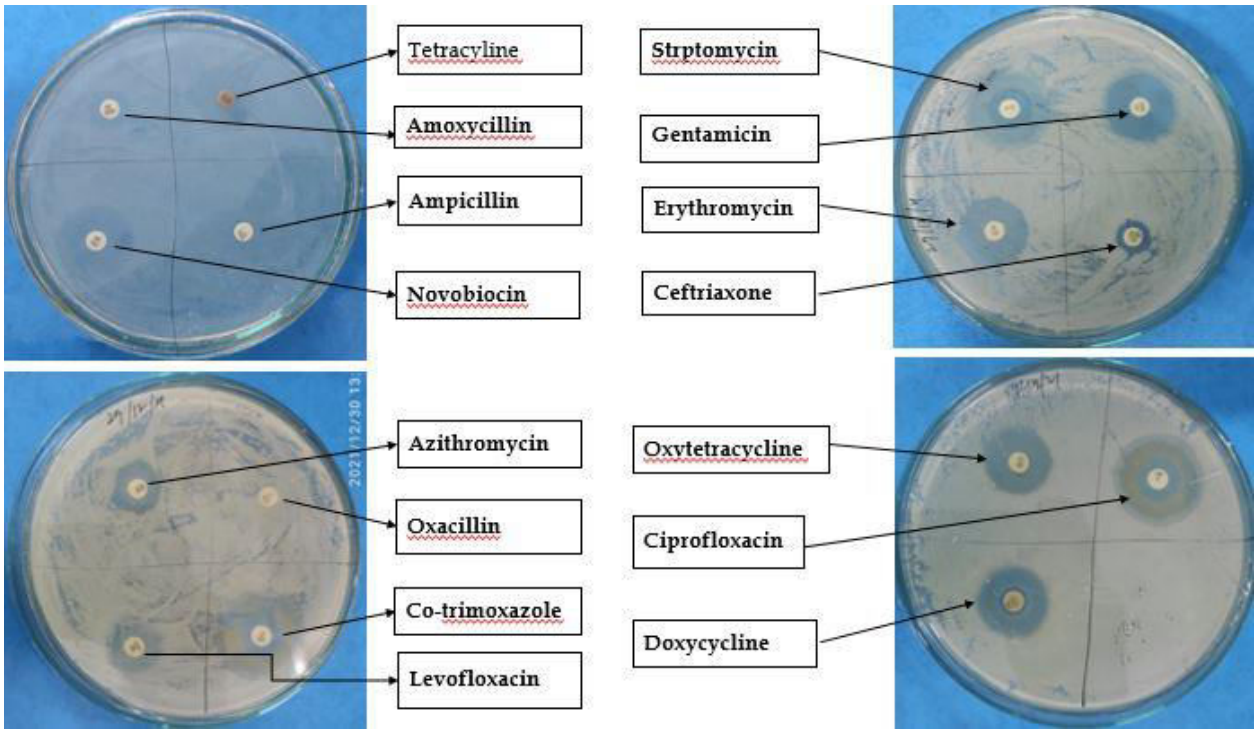


Figure.11 Antibiogram Study of *Aeromonas veronii* isolated from diseased *M. cavasius*



Bacteria was isolated from the tilapia kidney utilizing 16S rRNA gene sequence and found *Aeromonas* sp. which was responsible for the mass mortality of cultured tilapia (Soto-Rodriguez *et al.,* 2013).

16S rRNA PCR amplification was used to confirm bacterial species from the fish samples and this method is also used to differentiate most *Aeromonas* species (Borrell *et al.,* 1997; Rahman *et al.,* 2021) and 80% of the samples were identified as *A. hyrdophila* by the 16S rRNA gene amplification. Similarly 78% of the infectious diseases caused by *Aeromonas* spp. were identified (Kim *et al.,* 2002). The presence of 56% *Aeromonas* spp. in fish samples was confirmed using the 16S rRNA gene (Hussain *et al.,* 2014).

Antibiotic Sensitivity of the Isolated Bacteria

Among 15 antibiotics the most effective antibiotic against *Aeromonas veronii* (strain BDGTS_01) is Streptomycin because the highest zone of inhibition (130 mm) appears due to Streptomycin. (Figure 11; Table 2). *Aeromonas veronii* was not affected by 3 antibiotics i.e. Oxaciline, Co-trimoxazole and Ampicillin, but the bacteria was found sensitive to 11 antibiotics e.g., Oxytetracycline, Tetracycline, Ciprofloxacin, Doxycycline, Levofloxacin, Novobiocin, Erythromycin, Amoxycillin, Azithromycin, Streptomycin and Gentamicin, and found resistant to Ceftriaxone (Figure 11; Table 2).

Antibiotic sensitivity of *Aeromonas hydrophila* isolate was studied by other scientists (Goni *et al.,* 2020; Hamom *et al.,* 2020; Parven *et al.,* 2020; Tartor *et al.,* 2021). All of the isolates tested positive for ampicillin and amoxicillin. The least resistant drugs were doxycycline and gentamicin. Ciprofloxacin was effective against most isolates.

The antibiotic susceptibility of *Aeromonas* was investigated and discovered that the bacterium sample was sensitive to ciprofloxacin but totally resistant to ceftazidime (Zaman and Khalequzzaman, 2013). *Aeromonas schubertii* was identified as vulnerable to rifampicin and anorfloxacin but resistant to kanamycin and neomycin (Ren *et al.,* 2019). Most of the *Aeromonas* isolates were sensitive to Imipenem 34%, Chloramphenicol 50%, gentamycin 66%, and ciprofloxacin 92% (Monir *et al.,* 2017; Dryden *et al.,* 2011; Nagar *et al.,* 2011; Palu *et al.,* 2006). Ciprofloxacin and Tetracycline give the best result against the bacterial disease of Nile tilapia (Hamom *et al.,* 2020). Because of environmental conditions, different isolation sources, and varying drug use from place to place, the sensitivity and resistance pattern of *Aeromonas* may differ (Nagar *et al.,* 2011).

Biofloc aquaculture system has been growing very fast in Bangladesh since last several years due to its high per unit production through stocking of fishes at high density in a limited land area. But due to the intensification in the Biofloc system disease outbreaks have been seen to impede productivity.

This study investigated pathogenic bacteria from diseased Tengra (*M. cavasius*) culture in Biofloc system, using molecular and biochemical techniques and found out effective antibiotics to control the isolated bacteria. Initially, based on the colony characteristics from both nutrient agar and R-S media, and biochemical characteristics the suspected pathogens were identified as *Aeromonas* spp.

Finally, the analyses of 16S rRNA gene sequences of the bacteria isolated from Tengra (*M. cavasius*) was confirmed as *Aeromonas veronii*. In phylogenetic analysis, the sequence of *A. veronii* clustered together with other *A. veronii* retrieved from GenBank.

Among the tested fifteen antibiotics, Steptomycin was found more effective against *A. veronii* followed by Ciprofloxacin and Gentamycin. The presence of this pathogenic bacteria was responsible for severe mortality in the Biofloc aquaculture systems in Bangladesh. The findings of this research will be helpful to identify specific bacteria and to control bacterial diseases of the Tengra (*M. cavasius*) by knowing the specific pathogen responsible for specific diseases and the most effective antibiotics. The sequences of the isolated bacteria will be deposited to GenBank of National Centre for Biotechnology Information (NCBI), USA. However, due to high mortality of fishes in Biofloc aquaculture system, farmers are losing their interest in this culture system. Therefore, concern authority should develop required health management policies and strategies to protect this fast growing aquaculture system in Bangladesh as well as in the Globe to fulfill the growing demand of the animal protein.

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Ethical statement

Animal Subjects - With Ethical Approval: "All animal care and experimental procedures were conducted following guidelines set by the Sylhet Agricultural University Animal Research Ethics Board (Approval No. SAU/FHM/Ethical Committee/2022-05). Efforts were made to minimize animal suffering and reduce the number of animals used."

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Data availability

Data Available within the Article or Supplementary Materials: "The data that support the findings of this study are included within the article (and its supplementary materials)."

Author Contribution

Jannatul Mawa: Investigation, formal analysis, writing—original draft. Anupoma Achariya: Validation, methodology, writing—reviewing. Md. Zobayer Rahman:—Formal analysis, writing—review and editing. Md. Abdullah-Al-Mamun: Investigation, writing—reviewing. Shamima Nasren: Resources, investigation writing—reviewing. M. M. Mahbub Alam: Validation, formal analysis, writing—reviewing.

Declarations

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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